

Application for United States Letters Patent

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For

Growth Factor Binding Molecules

By

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GROWTH FACTOR BINDING MOLECULES

1. REFERENCES

This application claims the benefit of priority from Provisional Application Serial No. 60/190,938, entitled "Growth Factor Binding Molecules," filed March 21, 2000, by Saïd M. Sebti and Andrew D. Hamilton, which is herein incorporated in its entirety by reference.

2. STATEMENT OF FEDERAL SUPPORT

The present invention is made in whole or in part with financial support from the Federal Government under grant CA78038 from the National Institutes of Health, National Cancer Institute, and United States Army grant DAMD17-99-1-9458. The Federal Government may have certain rights in this invention.

3. FIELD OF INVENTION

The present invention relates generally to a family of synthetic molecules capable of binding to growth factors. More particularly, the invention relates to agents that bind growth factors through a plurality of peptide loops attached to a non-peptide, organic scaffold. The invention further relates to compositions comprising molecules that bind growth factors and their use in the treatment of mammals, where inhibition of cell growth and/or inhibition of angiogenesis are desirable, including the treatment of tumors and other diseases associated with hyper- or abnormal cell proliferation such as restenosis.

4. BACKGROUND

Vascular endothelial growth factor (VEGF), acidic fibroblast growth factor (aFGF), platelet-derived growth factor (PDGF), and insulin-like growth factor-1 (IGF-1) are active modulators of cell proliferation, with profound effects on tumor cell growth, and, in the case of PDGF, intimal smooth muscle cell growth. In addition, certain such growth factors stimulate blood vessel formation, or angiogenesis. Inhibition of angiogenesis is an important therapeutic goal in the control of tumor development in cancer patients.

Vascular disease treatment by balloon angioplasty, implantation of a stent, or by resection (e.g. a vascular graft), results in local damage to the endothelial cell lining of the

vessel wall. A result of the damage is that the smooth muscle cells of the intima begin to proliferate in a PDGF-dependent process termed intimal hyperplasia or restenosis. Despite this side-effect, percutaneous transluminal coronary angioplasty, stent insertion, and vascular by-pass and grafting remain primary treatments for many patients with coronary artery disease and can relieve myocardial ischemia by reducing lumen obstruction and improving coronary flow. A very significant proportion of patients develop restenosis within three months. Restenosis results in significant morbidity or frequently necessitates further interventions such as repeat angioplasty or coronary bypass surgery.

Platelet-derived growth factor (PDGF) is a potent inducer of growth and motility in several cell types such as fibroblasts, endothelial cells and smooth muscle cells. It induces cell proliferation, angiogenesis, wound healing, chemotaxis and inhibits apoptosis. PDGF has also been directly implicated in malignant diseases involving uncontrolled cell proliferation such as cancer where overexpression of PDGF and/or PDGF receptors is common in human tumors including glioblastomas and sarcomas. In addition to its role in stimulating uncontrolled growth of cancer cells, PDGF also stimulates the proliferation and migration of endothelial cells leading to formation of new blood vessels, a process that is required for the growth of tumors. Furthermore, PDGF has also been shown to stimulate endothelial cells to express high levels of vascular endothelial growth factor (VEGF) a potent angiogenesis inducer. Finally, the importance of PDGF in angiogenesis has also been documented by the fact that mice deficient in PDGF-BB or its receptor β are defective in blood vessel development.

PDGF elicits the above biological response by binding to its cell surface receptor, PDGFR, a tyrosine kinase. Binding results in dimerization, receptor auto (cross) phosphorylation and recruitment via the resulting phosphotyrosine of SH2 domain-containing signaling proteins such as Grb2/SOS1, PLC- γ , PI-3kinase and Src. These proteins trigger several arms of the PDGF signal transduction pathways that are involved in different cellular responses. For example, Grb2/Sos1 activates the GTPase Ras which in turn results in the activation of a cascade of mitogen-activated protein kinases (MAPK), such as Erk1 and Erk2, and this contributes to the proliferation arm of the PDGF signaling pathway. Others such as PI3-kinase activate yet another kinase, AKT, which is responsible for the survival or the anti-apoptotic arm of PDGF signaling pathways.

The involvement of PDGF overactivity in several diseases with excessive proliferation prompted many researchers to target PDGF as a therapeutic strategy. The

approaches that have been used to interfere with aberrant PDGF function in disease include PDGFR antibodies that block PDGF binding, linear or cyclic peptides corresponding to areas of PDGF that bind its receptor, receptor dimerization antagonists, and inhibitors of the tyrosine kinase activity of the receptor. An area that has been underexploited in the search of antagonists of PDGF overactivity is that of rationally designed agents that bind to PDGF and prevent it from activating its receptor. In this area only a few strategies such as antibodies against PDGF, soluble forms of PDGFR and DNA aptamers have been tried and those have had only marginal success. This is primarily due to the difficulty of designing agents to disrupt protein-protein interactions that are mediated over large surface binding areas, and because such molecules are often swiftly degraded by enzymes.

An attractive approach to producing synthetic molecules capable of binding to defined biological targets, involves synthetic antibody mimics disclosed by Hamilton and Yoshitomo in U.S. Patent 5,770,380, issued June 23, 1998, and which is hereby incorporated in its entirety by reference. Such molecules have the advantages of biological stability and can be rationally designed and screened. However, no such antibody mimics have previously been produced that specifically bind growth factors.

Here we describe a novel approach that targets growth factor surfaces, such as the regions of PDGF (loops I and III) that are involved in binding to its receptor, to overcome the aforementioned limitations in the art. These advantages and more will be apparent to those of skill in the art upon reading the following disclosure.

5. SUMMARY OF THE INVENTION

It is therefore an object of the present invention to provide growth factor binding compounds having a central non-peptide organic scaffold, such as a calix[4]arene, to which are attached two or more peptide loop domains. The peptide loop component is preferably based on a cyclic hexapeptide in which two residues are replaced by a 3-aminomethyl benzoate dipeptide mimetic containing a 5' amino substituent for attachment to the scaffold. In certain embodiments, the resulting molecules contain a functionalized and variable surface approximately 500 Å² (square Angstroms) in area. Such compounds are hereinafter referred to as growth factor binding molecules or agents.

It is a further object of the present invention to provide a growth factor binding molecule having an organic scaffold that is a calix[4]arene structure with specificity for

binding to platelet derived growth factor, vascular endothelial growth factor, or acidic fibroblast growth factor, insulin-like growth factor 1.

It is a further object of the present invention to provide a composition for inhibiting cellular proliferation in a subject in need thereof, the composition comprising a
5 pharmaceutically acceptable salt of a growth factor binding molecule of the present invention.

It is a further object of the present invention to provide a composition for treatment of a subject with excessive angiogenesis, in a subject in need thereof, the composition comprising a pharmaceutically acceptable salt of a growth factor binding molecule of the present invention.

10 It is a further object of the present invention to provide a method of treatment of a subject that has a tumor, the method comprising in a subject in need thereof, the composition comprising a pharmaceutically acceptable salt of a growth factor binding molecule of the present invention. wherein the effective amount inhibits growth of the tumor.

15 It is still a further object of the present invention to provide a method for measuring the amount of platelet-derived growth factor in a sample comprising obtaining a fluid sample, contacting the fluid sample with a growth factor binding molecule of the present invention and detecting the binding of the growth factor to the growth factor binding molecule of the present invention.

20 It is yet a further object of the present invention to provide a method for the delayed release of a platelet derived growth factor in a subject, the method comprising administering to the subject a stoichiometric complex of the growth factor and the growth factor binding molecule of the present invention.

25 It is yet a further object of the present invention to provide a means and composition for treating, inhibiting, or preventing restenosis or hyperplasia in a subject in need thereof, the composition comprising a pharmaceutically acceptable salt of a growth factor binding molecule of the present invention.

6. BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 illustrates the structure of embodiments growth factor binding (GFB) molecules.

Figures 2A & 2B illustrate the binding of GFB-111 to platelet derived growth factor and other growth factors known to be involved in aberrant cell growth and proliferation.

Figures 2C & 2D illustrate GFB-111 inhibition of [125 I]-PDGF binding to its receptor on NIH3T3 cells.

Figure 3 illustrates GFB-111 selectively inhibits PDGF-stimulated receptor tyrosine autophosphorylation and activation of MAPK.

Figure 4 illustrates GFB-111 selectively inhibits PDGF-stimulated DNA synthesis.

Figure 5 illustrates GFB-111 inhibits tumor growth and angiogenesis in nude mice.

Figure 6 illustrates the structure of calix tails.

Figure 7 illustrates the structure of GFB-114.

Figure 8 illustrates the structures of GFB-124.

Figure 9 illustrates the structures of GFB-126 and GFB-127.

Figure 10 illustrates the structure of "DPM-2" compounds (GFB-128 through GFB-131).

Figure 11 illustrates the structures of GFB-132 through GFB-137.

Figure 12 illustrates the inhibition of VEGF-stimulated flk-1 tyrosine phosphorylation by GFB-116.

Figure 13 illustrates selective inhibition of MAP kinase activation in vitro by GFB-116.

Figure 14 illustrates inhibition of human tumor growth in nude mice by GFB-116.

Figure 15 illustrates inhibition of angiogenesis by GFB-116.

Figure 16 illustrates inhibition of B-16 melanoma tumor growth in immune-competent mice by GFB116.

7. DETAILED DESCRIPTION OF SELECTED DRAWINGS

Figure 1 illustrates the structure of embodiments of growth factor binding compounds.

Figures 2A and 2B illustrate the binding of GFB-111 to PDGF. GFB-111 is incubated with increasing concentrations of PDGF-BB and the mixtures are then loaded onto native PAGE. Bands corresponding to the boxed area are cut out of the lanes of the gel, denatured with SDS-PAGE loading buffer, loaded onto a 4% SDS-PAGE gel and silver stained as described under Methods. Lanes 1-6 correspond to GFB-111 from the native gel; PDGF + GFB-111 standards; GFB-111/PDGF complex from the native gel; PDGF standard; GFB-111 standard and buffer, respectively. Data are representative of three independent experiments.

Figures 2C and 2D illustrate GFB-111 inhibiting [125 I]-PDGF binding to its receptor on NIH 3T3 cells. NIH 3T3 (PDGF) or NIH 3T3 cells overexpressing either EGFR (EGF), IGF1R (IGF) or Flk-1 (VEGF), are incubated with [125 I]-PDGF, [125 I]-EGF, [125 I]-IGF or [125 I]-VEGF, respectively and increasing concentrations of GFB-111 (◆) (C and D) or GFB-116 (■) (C). Cells are incubated at 4°C degrees for 1-3 hours and washed 3 times with PBS and three times with Tris 25mM pH8.0, 1%TX-100, 10% Glycerol, 1%SDS. Lysates plus wash are counted. Excess of cold growth factors is used to obtain non-specific binding levels. Data are representative of three independent experiments.

Figure 3 illustrates the manner in which GFB-111 inhibits selectively PDGF-stimulated receptor tyrosine autophosphorylation and activation of MAPK. A. Starved NIH 3T3 cells (PDGF-BB and PDGF-AA), NIH 3T3 cells overexpressing either EGFR (EGF) or Flk-1 (VEGF) are pre-treated with GFB-111 before stimulation with PDGF-BB, PDGF-AA, EGF and VEGF. Cell lysates are run on SDS-PAGE gels, then transferred to nitrocellulose and Western blotted with anti-phosphotyrosine (4G10) or anti-phosphorylated Erk1/Erk2 (New England Biolabs). B. Starved NIH 3T3 cells (PDGF-AA, PDGF-BB, aFGF, bFGF), NIH 3T3 cells overexpressing EGFR, IGF1R or Flk-1 and the human breast carcinoma SkBr3 that overexpresses ErbB2 (HRGβ) are pretreated with GFB-111 (100μM) for 5 min before 10 min stimulation with the indicated growth factors as described under Methods. Cells lysates are then immunoblotted with anti-phospho Erk1/Erk2 as described for A. Data are representative of 3 independent experiments.

Figure 4 illustrates GFB-111 selectively inhibiting PDGF-stimulated DNA synthesis.

Starved NIH 3T3 or NIH 3T3 overexpressing IGFR are treated with GFB-111 and either PDGF or IGF-1, respectively, then labeled with ^3H -thymidine. Cells are then washed, lysed and spotted onto glass fiber filters and ^3H -thymidine incorporation quantified by scintillation counting as described under Methods. Data are representative of 3 independent experiments.

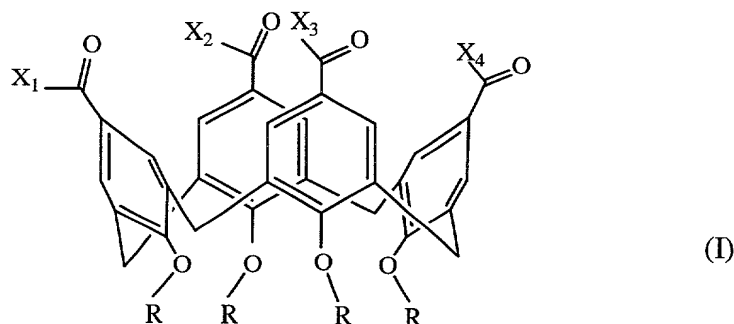
5 Figure 5 illustrates GFB-111 inhibiting tumor growth and angiogenesis in nude mice. A. GFB-111 inhibits the growth of the human glioblastoma U87MG in nude mice. U87MG cells are injected s.c. into nude mice and when tumors reached about 100 mm^3 , animals are dosed i.p. with 0.2ml once daily. Control animals received a saline vehicle whereas treated animals are injected with GFB-111 (50, 100 or 200mg/kg/day). The tumor volumes are
10 determined as described under Methods, *infra*. For detection of phosphorylated/activated Erk1/Erk2 in tumors in vivo, the tumors are extracted, rinsed and tissued in HEPES lysis buffer as described under Methods, *infra*. The lysates (50-100ug) are electrophoresed on a 15% SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted, with anti-phospho-p44/p42 MAPK (Thy202/Tyr204). Data are representative of 3 independent experiments. B. GFB-111 inhibits angiogenesis. On the indicated termination day of each experiment, the tumors are extracted as described under Methods. Four micrometer-thick tissue sections are obtained from the paraffin blocks and stained with hematoxylin and eosin (H&E) using standard histologic techniques. Tissue sections are also subjected to immunostaining for Factor VIII using the avidin biotin peroxidase complex technique. Rabbit polyclonal FVIII antibody is used at 1:400 dilution, following microwave antigen retrieval. Data are representative of two independent experiments.

8. DETAILED DESCRIPTION OF THE INVENTION

25 The agents disclosed herein bind growth factors. For example, in a specific embodiment, the growth factor to which an agent of the present invention binds is platelet derived growth factor (PDGF), and binding inhibits: activation of the PDGF receptor; stimulation of PDGF receptor tyrosine phosphorylation; activation of MAP kinase; tumor growth; and angiogenesis. Certain of these results are described by Blaskovich, M. A., et al. (2000) "Design of GFB-111, a platelet-derived growth factor binding molecule with
30 antiangiogenic and anticancer activity against human tumors in mice," Nature Biotechnology, Vol. 18, pp. 1065-1070, which is hereby incorporated in its entirety by reference.

The growth factor binding molecules of the present invention comprise a plurality of peptide loops attached to a non-peptide, organic scaffold. As used herein, the term “organic scaffold” is understood to mean a carbon-containing molecule capable of attachment to a plurality of peptide loops such that peptide loops so attached are substantially available to bind to a target compound, such as a growth factor, and the plurality of said loops thereby form a portion of the molecule capable of binding to a growth factor. In preferred embodiments, the organic scaffold is capable of binding 2, 3, or 4 peptide loops, which can be identical or non-identical. In preferred embodiments, the organic scaffold is a calixarene or (C₈-C₁₂)aryl (FIG.8). In most preferred embodiments, the organic scaffold is a non-cyclic calix[2]arene (FIG.10) or a cyclic calix[4]arene (FIG.4). It is not necessary that each calixarene unit or each available substitution position of a (C₈-C₁₂)aryl be attached to a peptide loop.

Thus, by way of example, in a preferred embodiment, growth factor binding compounds or agents are of the general structure of Formula I:



where R is substituted or unsubstituted (C₁-C₁₂)alkyl, (C₇-C₁₈)aralkyl, (C₆-C₁₈)aryl, (C₁-C₁₂)alkenyl, (C₇-C₁₈)aralkenyl, (C₁-C₁₂)alkylether, or the like. In certain embodiments, R is *n*-butyl, *n*-propyl, benzyl, or an alkyl ether such as O-3 or O-4 as illustrated in FIG. 6. In these preferred embodiments, X₁, X₂, X₃, and X₄ comprise different or identical cyclic peptide loops of the general structure of Formula II.

achieved by substitution in the 5-position by a nitro group which is then converted during the synthesis to an amino group for attachment to the calixarene. The synthesis of the cyclic peptides involves either solid phase chemistry or solution phase chemistry using a Kaiser oxime carboxylate activating group, discussed in greater detail in U.S. Patent 5,770,380 to Hamilton, et al., issued June 23, 1998, which is hereby incorporated in its entirety by reference.

Synthesis of the growth factor binding agents is carried out for both cyclic peptide and calixarene. Final coupling involves conversion of the calixarene tetraacid to the corresponding tetraacid chloride followed by reaction with the cyclic peptide amine derivative. This strategy allows the user to easily prepare large libraries of growth factor binding agents based on cyclic peptides with different sequences of four amino acids in the loop. Thus for 8 different peptide loop structures a total of 1,044 different growth factor binding agents will be available. By increasing the number of different peptide loops in the synthesis the diversity of artificial combining site structures can increase dramatically.

The structure thus synthesized, as illustrated in FIG.1, shows clearly the formation of a well-defined growth factor recognition site formed by the four peptide loop components. The power of this strategy is that a large number of growth factor binding agents can be prepared by the synthetic strategy and these can be screened for high affinity binding to a range of different growth factor targets. The potential applications include substitution of synthetic growth factor binding agents into much of the current antibody technology (immunosensors, diagnostic kits, etc.). The increased stability of these compounds has further application in therapeutics as well as the identification of catalysts that can function under extreme conditions.

Accordingly, a preferred method of practicing the present invention involves screening growth factor binding agents of the present invention. In accordance with this method, a library consisting of a large number of different growth factor binding agents is provided, each of the growth factor binding agents comprising a structure as described herein, preferably an organic scaffold to which is linked a plurality of peptide loops. Each growth factor binding agent in the library differs from each of the other growth factor binding agents in the library by having a different combination of peptide loops relative to the other growth factor binding agents in the library. Once the library of growth factor binding agents is provided, each of the growth factor binding agents in the library is capable of being screened

for growth factor binding agent effectiveness relative to other growth factor binding agents in the library by determining a binding affinity of the particular growth factor binding agent in the library being screened with respect to a growth factor relative to a binding affinity of one or more other growth factor binding agents in the library to that same target antigen.

5 Additionally, the library may be used by focusing on a particular growth factor binding agent and screening that growth factor binding agent for binding affinity with respect to a number of different target growth factors.

Referring again to Structure I, the scaffold comprises a calixarene ring wherein each of the arene groups that are part of the calixarene ring is linked to a peptide loop X via a
10 linking unit, preferably a 3-aminomethylbenzamide group, such as 3-aminomethyl-5-aminobenzamide (see FIG. 1) and each of said 3-aminomethylbenzamide groups is linked to the peptide loop. Of course, other combinations, other linking groups, other organic complexes, and other peptide loop structures are possible as will now be readily apparent to those of ordinary skill in the art. Examples of other possible linking units include 3-aminophenylacetamide and 7-aminonaphthyl-1-carboxamide.

Referring now to Structure II, R_1 , R_2 , R_3 , and R_4 , represent amino acid sidechains or derivatized or analogs of amino acid sidechains. Thus, in certain embodiments, R_{1-4} are sidechains of naturally occurring amino acids, such as lysine, arginine, histidine, phenylalanine, serine, alanine, leucine, tyrosine, tryptophan, glutamic acid, glutamine, aspartic acid, asparagine, isoleucine, valine, methionine, threonine and cysteine. It will be appreciated by one of ordinary skill in the art that R_{1-4} can also comprise modified or unusual amino acid sidechains, such as those disclosed in WIPO Standard ST.25 (1998), Appendix 2, Table 4A, or can comprise labeled moieties such as fluorescent labels or radiolabels. It will further be appreciated that the amino acids comprising the peptide loops can also be D-amino
25 acids, L-amino acids, or both within a single peptide loop. In the present invention, a peptide loop rather than an open-ended peptide chain is preferred because such loops are more chemically stable than open-ended chains.

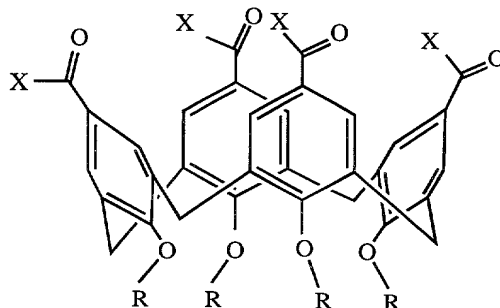
In specific embodiments of the present invention, a calixarene scaffold is used because its ring structure and non-protein nature give it stability in extreme environmental
30 conditions and in the presence of protease enzymes. In addition, its ring structure, as detailed below, helps force the attached peptide loops to come into close proximity to each other,

mimicking the binding site of an antibody. Finally, the arene groups of the calixarene rings may be attached at their number four positions to alkyloxy groups.

The above-mentioned alkyloxy groups are important in the preferred embodiment of the present invention because they enforce the rigid three-dimensional conformation of the calixarene scaffold. In two dimensions, calixarene is a ring, however, in three dimensions the ring is seen to have a cup-like conformation, as is illustrated in FIG.1. The side of the cup from which the alkyloxy groups project, that is, the "4" positions of the arene groups, may be called the bottom of the cup. Thus the opposite side, which is the side attached to the peptide loops, that is, the "1" positions of the arene groups, may be called the top of the cup. Because the alkyloxy groups (OR) attached to the bottom of the cup reenforce the cup conformation, all of the peptide loops X at the top of the cup are forced into close proximity to one another. This close proximity of the peptide loops, in turn, mimics the structure of an antibody and forces all of the peptide loops to be available for contact with and binding to an antigen of interest.

In Formula II, R₁, R₂, R₃, and R₄, are each amino acid side chains that define a tetra-peptide sequence in which the tetra-peptide is covalently attached at the amino and carboxy termini of the tetra-peptide to a 3-aminomethyl-5-aminobenzamide linking group. In certain embodiments, for example PDGF-binding embodiments, the tetra-peptide sequence is selected from the group consisting of GDFD, GDDD, D-ADGD (in which the alanine is in the D conformation, other amino acids being in the L conformation), GDLD, GDAD, GDGY, ADGD, GDSD, GKGF, GK GK, GDND, PDGD, GDDG, GDDY, and functionally related derivatives thereof. In this embodiment, the preferred R group is *n*-butyl.

In an alternative embodiment, the growth factor binding compound has the structure of Formula III:



(III)

Here, X comprises attached identical cyclic peptide loops as illustrated by structure II, wherein R₁, R₂, R₃ and R₄, are each amino acid side chains that define the tetrapeptide sequence, in which the tetrapeptide is covalently attached at the amino and carboxy termini of the tetrapeptide to a 3-aminomethyl-5-aminobenzamide.

It will be apparent to those of ordinary skill that not every substitutable position of the organic scaffold need be attached to a peptide loop. Thus, embodiments having a calix[4]arene organic scaffold with fewer than 4 peptide loops attached to it are envisaged as within the scope of the present invention (see, for example, FIG. 9). It will further be appreciated that numerous permutations of both the positioning of specific peptide loops, and the corresponding unsubstituted positions within the organic scaffold, are possible within the present invention.

A pharmaceutical composition is formed by means such as known by those skilled in the art, wherein, for example, the compound of Formula I, or Formula III, or a salt thereof, or a combination thereof, is incorporated into a pharmaceutically acceptable carrier. These pharmaceutical compositions can be used for treatment of a subject who suffers from excess cellular proliferation, including restenosis, excess angiogenesis, a tumor, a condition comprising undesirable angiogenesis, or a combination thereof. Applications in both veterinary and human use are indicated.

Where a subject has one or more tumors, treatment can comprise administering to the subject an effective amount of a composition comprising, for example, Formula I or Formula III or their respective salts, and a pharmaceutically acceptable carrier. The compositions are suitable for the treatment of all tumors requiring activation by growth factors or angiogenesis for tumor growth, such as glioblastomas, adenosarcomas, and sarcomas. Further examples of tumors treatable through the present invention, include, but are not limited to:

paranasal sinus, nasopharynx, oral cavity and oropharynx squamous cell carcinomas and adenocarcinomas; oral lymphomas; adenoid cystic carcinoma; paragangliomas; squamous cell carcinoma, adenocarcinoma, large cell (undifferentiated)

carcinoma , and small cell carcinoma of the lung; mediastinal thymomas, lymphomas and neurogenic carcinomas; squamous cell carcinoma and adenocarcinoma of the esophagus; adenocarcinoma of the stomach; ductal adenocarcinoma, mucinous cystadenocarcinoma, acinar cell carcinoma, unclassified large cell carcinoma, small cell carcinoma, and

5 pancreatoblastoma of the pancreas; hepatocellular carcinoma, hepatoblastoma, cholangiocarcinoma, cholangiocellular carcinoma, cystadenocarcinoma, squamous cell carcinoma, angiosarcoma, hemangioendothelioma, leiomyosarcoma, malignant schwannoma, fibrosarcoma, malignant fibrous histiocyoma, lymphoma, osteosarcoma, rhabdomyosarcoma and mesenchymal sarcoma of the liver; adenosarcoma, squamous cell carcinoma,

10 adenosquamous carcinoma, oat cell carcinoma (small cell), carcinosarcoma, malignant lymphoma, malignant melanoma, rhabdomyosarcoma, fibrous histiocyoma, and angiosarcoma of the gallbladder; adenocarcinoma, fibrosarcoma, leiomyosarcoma, liposarcoma, angiosarcoma, lymphangiosarcoma, lymphoma, and neurofibrosarcoma of the small bowel; adenocarcinoma, mucinous adenocarcinoma, signet-ring cell adenocarcinoma, squamous cell carcinoma, adenosquamous carcinoma, undifferentiated carcinoma, unclassified carcinoma, argentaffin carcinoid tumor, nonargentaffin carcinoid tumor, composite carcinoid tumor, and leiomyosarcoma of the large intestine; squamous cell carcinoma, transitional (cloacogenic) carcinoma, adenocarcinoma, papillary villous carcinoma, and mucinous adenocarcinoma of the anus; renal clear cell, granular cell and sarcomatoid carcinoma; nephroblastoma; transitional cell carcinoma of the ureter; carcinoma of the bladder; adenocarcinoma of the prostate; squamous cell carcinoma and

20 adenocarcinoma of the cervix; adenocarcinoma, cystadenocarcinoma, carcinoma, and adenofibroma, cystadenofibroma, sarcoma, and adenoacanthoma of the ovary; papillary, follicular, and medullary carcinoma of the thyroid; adenoma and carcinoma of the parathyroid; adenoma and carcinoma of the adrenal cortex; fibrosarcoma, and fibrous histiocyoma, liposarcoma, squamous cell carcinoma, and sarcoma of soft tissues; leiomyosarcoma, and rhabdomyosarcoma of muscle tissues; osteogenic sarcoma, fibrosarcoma, fibrous histiocyoma and chondrosarcoma of bone; angiosarcoma, lymphangiosarcoma, and Kaposi's sarcoma; malignant glomus tumor and

25 hemangiopericytoma; synovial sarcoma and giant cell tumor of the tendon sheath; malignant peripheral nerve sheath tumor, neurofibrosarcoma, malignant triton tumor, malignant glandular schwannoma, epithelioid schwannoma, malignant granular cell tumor, nerve clear

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cell sarcoma, and malignant paraganglioma; chondrosarcoma and osteosarcoma; and malignant mesenchymoma.

The compositions are effective over a wide range of concentrations. The effective amounts are those suitable to bring the concentration in the biological fluid of the subject to between about 1 picomolar and about 1 millimolar, preferably between about 10 nanomolar and about 10 micromolar, most preferably about 0.2 micromolar. Alternatively, the effective amount can be expressed in terms of the body weight of the subject. An amount effective to treat the disease is between about 0.3 micrograms per kilogram of body weight to about 330 milligrams per kilogram of body weight. Most preferably a concentration of about 1.0 milligrams per kilogram is suitable.

Some tumors express elevated amounts of platelet derived growth factor and/or platelet derived growth factor receptor. To measure the amount of a platelet derived growth factor in a sample, the growth factor binding compound can be radiolabeled, fluorescently labeled, labeled with a reporter enzyme, or some combination thereof. In addition, the growth factor binding compound can covalently or noncovalently attach to a surface or a particle. The covalent linkage can be through an amine linkage, an amide linkage, an ester linkage, or an ether linkage. The surface or particle can comprise of polystyrene, polyvinyl chloride, glass, ceramic, iron, or any other suitable material.

A method of delayed release of a growth factor is, for example, to administer to the patient a stoichiometric complex of the growth factor bound to its growth factor binding agent. The rate of release of growth factor is then determined by the unbinding rate constant for the complex. The growth factor of the complex can comprise variant forms of growth factor, such as, for example, xenogenic forms of platelet derived growth factor, or chemically derivatized versions of platelet derived growth factor.

9. EXAMPLES

The following specific examples are provided to better assist the reader in the various aspects of practicing the present invention. As these specific examples are merely illustrative, nothing in the following descriptions should be construed as limiting the invention in any way. Such limitations are, of course, defined solely by the accompanying claims.

9.1 Materials and Methods

Inhibition of PDGF-dependent receptor tyrosine phosphorylation by growth factor binding agents.

Starved NIH 3T3 cells are pretreated with various growth factor binding agents (0-100 μ M) for 5 min before stimulation with PDGF (10 ng/mL) for 10 min. The cells are then harvested and lysed, and the proteins from the lysates separated by SDS-PAGE and immunoblotted with an anti-phosphotyrosine antibody (4G10: Upstate Biotechnology, Lake Placid, NY). The phosphotyrosine PDGF receptor bands are scanned, and the concentrations of growth factor binding molecules that inhibited the ability of PDGF to stimulate PDGFR tyrosine phosphorylation by 50% are determined.

PDGF binding

GFB-111 (50 μ M) is incubated with buffer A (PBS 0.5% BSA) or PDGF-BB (1, 3 and 4 μ M in buffer A) at room temperature for 10 minutes. The mixtures are then loaded onto a native 4% PAGE. The gel is then stained with silver nitrate. Duplicate lanes are run in the same gel but are not stained. Bands corresponding to the boxed area are cut out of the lanes of the gel that is not stained, denatured with SDS-PAGE loading buffer (Tris 100mM pH6.8, 15% Glycerol, 3% SDS, 7.5% b-mercaptoethanol, 0.05% bromophenol blue), loaded onto a 4% SDS-PAGE gel and silver stained.

Binding of [125 I]-PDGF to NIH 3T3 cells: inhibition by GFBs.

NIH 3T3 (PDGF) or NIH 3T3 cells overexpressing either EGFR (EGF), IGFR (IGF) or Flk-1 (VEGF), are incubated with [125 I]-PDGF, [125 I]-EGF, [125 I]-IGF or [125 I]-VEGF, respectively (50,000 cpm per well) and increasing concentrations of GFB-111 or GFB-116. Cells are incubated at 4°C for 1-3h. After discarding media, the cells are washed 3 times with PBS and three times with Tris 25mM pH 8.0, 1% T-X-100, 10% glycerol, 1% SDS. Lysates plus wash are counted. Excess of cold growth factors is used to obtain non-specific binding levels.

PDGF-stimulated receptor tyrosine autophosphorylation and activation of MAPK.

Starved NIH 3T3 cells (PDGF-BB and PDGF-AA), NIH 3T3 cells overexpressing either EGFR (EGF) or Flk-1 (VEGF) are pre-treated with the indicated concentrations of GFB-111 for 5 minutes before 10 minutes of stimulation with PDGF-BB (10ng/ml), PDGF-AA (10ng/ml), EGF (100ng/ml) and VEGF (50ng/ml). Cell lysates are run on SDS-PAGE gels, then transferred to nitrocellulose and Western blotted with anti-phosphotyrosine (4G10) or anti-phosphorylated Erk1/Erk2 (New England Biolabs).

In other experiments, starved NIH 3T3 cells (PDGF-AA, PDGF-BB, aFGF, bFGF), NIH 3T3 cells overexpressing EGFR, IGF-1R or Flk-1 and the human breast carcinoma SkBr3 that overexpresses ErbB2 (HRG β) are pretreated with GFB-111 (100 μ M) for 5 min before 10 min stimulation with the indicated growth factors at the concentrations shown above and aFGF (10ng/ml), bFGF (50ng/ml), IGF-1 (50ng/ml) and HRG β (10ng/ml). Cell lysates are then immunoblotted with anti-phospho Erk1/Erk2 as described above.

PDGF-stimulated DNA synthesis.

Starved NIH 3T3 or NIH 3T3 overexpressing IGFR are treated for 16 hours with indicated concentrations of GFB-111 and either 10ng/ml PDGF or 50ng/ml IGF-1, respectively, then labeled for 2 hr (NIH 3T3) or 4 hr (IGFR) with ³H-thymidine. Cells are then washed in PBS, lysed in 0.1N NaOH and spotted onto glass fiber filters. DNA is precipitated with ice cold ethanol then ³H-thymidine incorporation quantified by scintillation counting.

Antitumor efficacy studies in nude mice.

U87MG cells (human glioblastoma) are harvested, resuspended in PBS and injected s.c. into the right and left flank (10 X 10⁶ cells per flank) of 8 week old female nude mice (Charles River, Wilmington, Massachusetts). When tumors reached about 100 mm³, animals are dosed i.p. with 0.2ml once daily. Control animals received a saline vehicle whereas treated animals are injected with GFB-111 (50, 100 or 200mg/kg/day). The tumor volumes are determined by measuring the length (l) and the width (w) and calculating the volume ($V=lw^2/2$) as described previously. For detection of phosphorylated/activated Erk1/Erk2 in tumors in vivo, the tumors are extracted 20 min after the last GFB-111 injection on day 32, rinsed and the tissue homogenized in HEPES lysis buffer (30mM Hepes, pH 7.5, 1% T-X-100, 10% glycerol, 10mM NaCl, 5mM MgCl₂, 25mM NaF, 1mM EGTA, 2mM Na₃VO₄, 10

μg/ml trypsin inhibitor, 25μg/ml leupeptin, 10μg/ml aprotinin, 2mM PMSF). The lysates (50-100μg) are electrophoresed on a 15% SDS-PAGE, transferred to nitrocellulose membranes and immunoblotted, with anti-phospho-p44/p42 MAPK (Thy202/Tyr204).

Angiogenesis studies.

On the indicated termination day of each nude mouse experiment described above, (see also Table 2), the tumors are extracted and half frozen at -70°C, and half is fixed in 10% neutral buffered formalin for 6 hrs. After fixation the tissue samples are processed into paraffin blocks. Four micrometer-thick tissue sections are obtained from the paraffin blocks and stained with hematoxylin and eosin (H&E) (Richard-Allan Scientific, Kalamazoo, MI) using standard histologic techniques. Tissue sections are also subjected to immunostaining for Factor VIII using the avidin biotin peroxidase complex technique (Vectastatin Elite ABC Kit, Vector, Burlingame, CA). Rabbit polyclonal FVIII antibody (Von Willebrand Factor, Dako Corporation, Carpinteria, CA) is used at 1:400 dilution, following microwave antigen retrieval (4 cycles of 5 minutes each on high in 0.1M citrate buffer).

EXAMPLE 1 PDGF BINDING AGENTS

9.2 Design of Molecules that Bind PDGF and Disrupt its Signaling Function

A novel series of protein surface binding agents are prepared that bind PDGF and disrupt its signaling function. The molecules are composed of a central calix[4] arene scaffold to which is attached four peptide loop domains (FIG.1). The peptide loop component is based on a cyclic hexapeptide in which two residues are replaced by a 3-aminomethylbenzoate dipeptide mimetic containing a 5-amino substituent for attachment to the scaffold. The resulting molecules (as shown in Table 1) contain a functionalized and variable surface approximately 500Å² in area. Several peptide loop sequences are synthesized (Table 1) to provide molecular surfaces with negatively and positively charged as well as hydrophobic regions designed to bind to complementary areas on the target growth factor. To identify PDGF binding molecules from this library, a cell-based screening assay is developed with NIH 3T3 cells to detect molecules capable of blocking PDGF-BB-induced tyrosine autophosphorylation of the PDGF receptor. The ability of the identified molecules to bind PDGF-BB and inhibit interaction with its receptor is then confirmed biochemically. Starved NIH 3T3 cells are pretreated for 5 min with the synthetic molecules prior to stimulation with PDGF for 10 min. The effects of the molecules on PDGF-stimulated

receptor tyrosine phosphorylation are determined by antiphosphotyrosine Western blotting as described in Table 1. GFB-111 is identified as one potential PDGF binder and as a potent ($IC_{50} = 250nM$) inhibitor of PDGF-BB-stimulation of PDGF receptor tyrosine autophosphorylation (Table 1). GFB-111 has four-fold symmetry containing 4 identical peptide loops with negative and hydrophobic residues, GlyAspGlyTyr (GDGY), that are designed to be complementary to the PDGF surface involved in binding to PDGFR. The area of PDGF-BB that binds PDGFR is composed primarily of positive and hydrophobic residues. Substitution of aspartic acid in the loop by positively charged lysine is poorly tolerated as can be seen by comparison of GFB-111 (GDGY) ($IC_{50} = 250nM$) to GFB-115 (GKGF) ($IC_{50} = 50\mu M$) and GFB-116 (GKGK) ($IC_{50} = 40\mu M$), indicating that negatively charged residues are important for the inhibitory activity of GFB-111. However, negatively charged residues alone are not sufficient and the presence of an aromatic hydrophobic residue in the loop increases affinity as can be seen by comparison of GFB-111(GDGY) with GFB-107 (GDGD) ($IC_{50} = 2.5\mu M$) as well as of GFB-122 (GDDY) ($IC_{50} = 1.7\mu M$) with GFB-106 (GDDD) ($IC_{50} = 8\mu M$) (Table 1).

Table 1. Inhibition of PDGF stimulated PDGFR Tyrosine Phosphorylation and Signaling in NIH 3T3 by GFBs

Designation	Scaffold	R ₁ -R ₄ sequence	(IC ₅₀) Inhibition (μM)	
			PDGFR-Y-P	p42/Erk2
GFB-101	O4-Calix ^A	4-GFGD	>500	>500
GFB-102	"	4-GDFD	>500	500
GFB-103	"	4-GDGD	>500	>500
GFB-104	O3-Calix ^A	4-GDGD	>500	>500
GFB-105	But-Calix ^A	4-GDFD	2.4	28
GFB-106	"	4-GDDD	8	33
GFB-107	"	4-GDGD	2.5	10
GFB-108	"	4-d-ADGD ¹	9	57
GFB-109	"	4-GDLL	7.5	15
GFB-110	"	4-GDAD	1.7	7
GFB-111	"	4-GDGY	0.25	2.6
GFB-112	"	4-ADGD	29	26
GFB-113	"	4-GDSD	2.8	30
GFB-114	"	(4-) 8-acid (See Figure 7)	>50	>50
GFB-115	"	4-GKGF	>50	>50
GFB-116	"	4-GKGK	40	28
GFB-117	"	4-GDND	5.8	36
GFB-118	"	4-D-2-NalDGD ²	0.7	1.2
GFB-119	"	4-PDGD	20	32

Designation	Scaffold	R ₁ -R ₄ sequence	(IC ₅₀) Inhibition (μM)		
			PDGFR-Y-P	p42/Erk2	
GFB-120	"	4-GDDG	1.3	1.7	
GFB-121	"	4-d-AbuDGD ³	5.6	8.5	
GFB-122	"	4-GDDY	1.7	19	
GFB-123	"	1-GDDG	>50	>50	
GFB-124	"	See Figure 8	>50	>50	
GFB-126	"	3 x GDDG (See Figure 9)	1.9	4.6	
GFB-127	"	2 x GDDG (See Figure 9)	32	>50	
GFB-128	"	DPM-2-GDGY (See Figure 10)	26	>50	
GFB-129	"	DPM-2-GDDD (See Figure 10)			
GFB-130	"	DPM-2-GDDY (See Figure 10)			
GFB-131	"	DPM-2-GDDG (See Figure 10)			
				p42	p44
GFB-132	But-Calix	3-GDGY-1-GDDG (See Figure 11)	0.4	2.9	3
GFB-133	"	3-GDGY-1-CO ₂ H (See Figure 11)	0.8	3.2	3
GFB-134	"	3-GDGY-1-GDDY (See Figure 11)	1.9	2.5	2.6
GFB-135	"	3-GDGY-1-GDDD (See Figure 11)	1.9	3.2	24
GFB-136	"	3-GDDD-1-GDGY (See Figure 11)	3	25	24
GFB-137	"	2-GDGY-2-GDDG (See Figure 11)	0.4	2.9	47

^A The difference between GFB-101 through GFB-103 (O4-Calix), GFB-104 (O3-Calix), and the rest of the GFB compounds (But-Calix) is related to the structure of the calix tail. (Please refer to Figure 6.)

¹ GFB-108: The "d" refers to D-amino acid, so d-ADGD is (D-Ala)-Asp-Gly-Asp.

² GFB-118: D-2 Nal is structurally related to D-Phe. Instead of the phenyl ring in Phe, it has a naphthelene ring linked through the 2-position.

³ GFB-121: d-Abu is D-aminobutyric acid. It has an ethyl group in the side chain while Ala has a methyl group in the side chain.

Starved NIH 3T3 cells are pretreated with the various GFBs (0-100 μM) for 5 min. prior to stimulation with PDGF (10 ng/ml) for 10 min. The cells are then harvested lysed and the proteins from the lysates are separated by SDS-PAGE and immunoblotted with an anti-phosphotyrosine antibody (4G10, Upstate Biotechnology, Lake Placid, NY). The phosphotyrosine PDGF receptor bands are scanned and the concentrations of GFBs that inhibit the ability of PDGF to stimulate PDGFR tyrosine phosphorylation by 50% are determined. Data for each compound is representative of at least two independent experiments. Figure 1 illustrates the structure of growth factor binders. GFBs are

synthesized as described by Park, H.D. et al., Protein surface recognition by synthetic receptors: A route to novel sub-micromolar inhibitors for chymotrypsin. *J. Am. Chem. Soc.* **121**, 8-13 (1999); and Hamuro, Yet al., A calixarene with four peptide loops: An antibody mimic for recognition of protein surfaces. *Angew. Chemie Int. Ed. Engl.* **36**, 2680-2683 (1997), each herein incorporated by reference in its respective entirety.

9.3 GFB-111 is a PDGF-BB Binding Molecule

The structure activity relationship results in Table 1 are consistent with GFB-111 being a PDGF-BB binding molecule. Whether GFB-111 binds PDGF-BB and also inhibits binding of [¹²⁵I] PDGF to its receptor on NIH 3T3 cells is determined by gel electrophoresis by mixing GFB-111 with PDGF-BB. This results in a concentration-dependent disappearance of GFB-111 which is paralleled by the appearance of a slower migrating band on native polyacrylamide gel electrophoresis (PAGE). In this native PAGE system, PDGF-BB itself migrates toward the cathode and hence does not penetrate the gel. To demonstrate that the slower migrating band is formed by the complex between GFB-111 and PDGF-BB, the band is cut out of the native gel, processed and loaded onto denaturing SDS-PAGE gel. Figure 2B illustrates that the GFB-111 standard (lane 5) as well as the GFB-111 band cut out of Figure 2A gel (lane 1) migrates on SDS-PAGE as a single band that elutes faster than the dye front. Their separation on SDS-PAGE (lane 2) is confirmed by mixing PDGF-BB standard (lane 4) and GFB-111 standard (lane 5). Note that the band cut out of the native gel presumed to contain the complex does indeed contain both PDGF-BB and GFB-111 (Figure 2B, lane 3). The interaction is structure-dependent since the positively charged derivative GFB-116 (Table 1) does not bind to PDGF-BB as demonstrated by gel electrophoresis. Furthermore, GFB-111 does not bind to the related peptide growth factors EGF (epidermal growth factor) and IGF-1 (insulin-like growth factor-1) demonstrating that GFB-111 is selective for PDGF.

GFB-111 is incubated with increasing concentrations of PDGF-BB and the mixtures are then loaded onto native PAGE. Bands corresponding to the boxed area are cut out of the lanes of the gel, denatured with SDS-PAGE loading buffer, loaded onto a 4% SDS-PAGE gel and silver stained as described under Methods. Lanes 1-6 correspond to GFB-111 from the native gel; PDGF + GFB-111 standards; GFB-111/PDGF complex from the native gel; PDGF standard; GFB-111 standard and buffer, respectively. Data are representative of three

independent experiments.

9.4 Complementarity of the recognition domain of GFB-111 with the receptor binding surface of all PGDF

The complementarity of the recognition domain of GFB-111 (negatively charged and hydrophobic residues) with the receptor binding surface of PDGF (positively charged and hydrophobic sites) suggests that GFB-111 blocks binding of PDGF to PDGFR. Whether GFB-111 can block [125 I]-PDGF-BB specific binding in NIH 3T3 cells is determined by pre-treatment of the PDGF-BB with increasing concentrations of GFB-111. Figure 2C illustrates that in the absence of GFB-111, [125 I]-PDGF-BB is bound by NIH3T3 cells. However, pre-treatment with increasing concentrations of GFB-111, results in a concentration-dependent decrease in PDGF-BB binding. The IC_{50} value of binding inhibition is 250nM (Figure 2C) which is similar to the IC_{50} value of GFB-111 inhibition of PDGF-BB-stimulated PDGFR tyrosine phosphorylation (Table 1). Figure 2C also shows that PDGF-BB binding to its receptor is blocked by GFB-111 but not by GFB-116. Similar experiments with EGF, IGF-1 and VEGF (vascular endothelial growth factor) demonstrate a lack of significant effects of GFB-111 (10 μ M) on [125 I]-EGF or [125 I]-IGF-1 binding but inhibition of [125 I]-VEGF binding by 50% in NIH 3T3 cells overexpressing EGFR, IGF-1R and VEGFR (Flk-1), respectively (Figure 2D).

9.5 Selectivity of GFB-111

The selectivity of GFB-111 is shown by its ability to block growth factor-stimulated tyrosine phosphorylation of several receptor tyrosine kinases and subsequent activation of two mitogen-activated protein kinases (MAPK), Erk1 and Erk2. Figure 3A shows that PDGF-BB-stimulation of starved NIH 3T3 cells in the absence of GFB-111 results in PDGFR autophosphorylation and Erk1/Erk2 activation. However, pre-treatment of these cells with GFB-111 (0.1 to 10 μ M) for 5 min results in a concentration-dependent inhibition of PDGF-BB stimulation of receptor tyrosine phosphorylation with an IC_{50} of 250nM and a complete block at 3 μ M GFB-111. Figure 3A also shows that GFB-111 is a potent inhibitor of PDGF-dependent activation of Erk1 and Erk2 (IC_{50} of 0.8 and 1.2 μ M, respectively). PDGF-AA stimulation of PDGF receptor phosphorylation and Erk1/Erk2 activation is also blocked with GFB-111 with a similar potency. In contrast, GFB-111 at concentrations as high as 100 μ M

does not affect the ability of EGF to stimulate EGFR tyrosine phosphorylation and Erk1/Erk2 activation. For comparison, PD158780, a known EGFR tyrosine kinase inhibitor, blocks EGFR tyrosine phosphorylation and Erk1/Erk2 activation (Figure 3A). GFB-111 is able to inhibit VEGF-stimulated Flk-1 tyrosine phosphorylation and MAPK activation but at concentrations higher ($IC_{50} = 10\mu M$) than those required for PDGF ($IC_{50} = 250nM$). Furthermore, IGF-1-, aFGF-, bFGF- and HRG β -activation of Erk1/Erk2 is minimally affected by GFB-111 at concentrations as high as $100\mu M$ (Figure 3B). Thus, GFB-111 blocks PDGF signaling at very low concentrations, inhibits VEGF signaling at higher concentrations ($10\mu M$), but has negligible effect on EGF, IGF-1, aFGF, bFGF and HRG β signaling at very high concentrations.

9.6 The Effectiveness of GFB-111 on PDGF-Induced Cellular Responses.

The selectivity of PDGF-induced cellular responses, is evaluated by the selectivity of GFB-111 to inhibit growth factor-stimulated DNA synthesis using a [3H]-thymidine incorporation assay. In NIH 3T3 cells PDGF-BB stimulated DNA synthesis by 10-fold and GFB-111 suppresses this stimulation in a dose dependent manner with an IC_{50} of $6.5\mu M$ (Figure 4). In contrast GFB-111 is ineffective at inhibiting IGF-1-stimulated DNA synthesis at concentrations as high as $100\mu M$ (Figure 4). These data further confirm the highly selective character of GFB-111 towards PDGF.

9.7 The Effect of GFB-111 on Angiogenesis and Human Cancer Cell Growth in Animal Models.

The ability of GFB-111 to potently and selectively block PDGF-dependent signaling and DNA synthesis, coupled with the ability of PDGF to stimulate angiogenesis and contribute to cancer cell growth, permits determination of *in vivo* anti-tumor efficacy of GFB-111 in the nude mouse human xenograft model. Figure 5A illustrates that daily treatment (i.p.) of nude mice implanted s.c. with the human glioblastoma U87MG with GFB-111 at 50, 100 and 200mg per kg results on day 32 in a dose-dependent inhibition of tumor growth of 56, 81 and 88%, respectively (Table 2). Figure 5a insert illustrates that extraction and processing of tumors on day 32, shows that Erk1/Erk2 activation is blocked in the tumors from the animals treated with 100 and 200 mg per kg GFB-111. The ability of GFB-111 to

inhibit tumor growth is not limited to U87MG. GFB-111 is also effective at inhibiting the tumor growth in nude mice of the human lung adenocarcinoma A-549 and the rat glioma C6 (Table 2). However, the growth of the human medulloblastoma DaOY is minimally affected at 50mg per kg per day GFB-111 (Table 2).

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Table 2. GFB-111 Antitumor Efficacy

Cell line	Tumor origin	% Tumor growth inhibition			
		Experiments			
		1	2	3	4
U87-MG	Human brain	61% (35) (50 mpk)	56% (32) (50 mpk)	81% (32) (100 mpk)	88% (32) (200 mpk)
A-549	Human lung	62% (30) (50 mpk)	53% (31) (50 mpk)	64% (27) (50 mpk)	N/D
C6	Rat brain	54%(15) (50 mpk)	64% (15) (50 mpk)	46% (18) (50 mpk)	N/D
DaOY	Human brain	21% (48) (50 mpk)	0% (37) (50 mpk)	N/D	N/D

9.8 Inhibition of Angiogenesis *in vivo* by GFB-111.

Because GFB-111 is effective at inhibiting the tumor growth of human cancer cells that express a PDGF/PDGFR autocrine loop (U87MG) as well as those that express neither PDGF nor PDGFR (A-549), and because PDGF promotes angiogenesis, the effect of GFB-111 on inhibition angiogenesis *in vivo* is shown. U87MG tumor biopsy specimens on day 32 are fixed and processed into paraffin blocks. Tissue sections are subjected to immunostaining with the vascular marker Factor VIII. Tumors from control animals show very marked staining. In contrast as illustrated in Figure 5B, the Factor VIII immunostaining is decreased in a dose-dependent manner in tumors from animals treated with 50, 100 and 200 mg per kg GFB-111. Quantification of microvessels at two field magnifications (250X and 400X) clearly demonstrates that GFB-111 inhibits angiogenesis strongly in U87MG and A-549 tumors, to a lesser degree in C6 tumors, and has no effect on angiogenesis in DaOY tumors (Table 3).

Table 3. GFB-111 Anti-angiogenic activity

Tumor	Group	Microvessel count (Mean±SD)	
		250X	400X
A-549	Control	15.66±7.00	7.74±2.88
	GFB-111 (50 mpk)	3.63±1.36	2.34±1.01
U87-MG	Control	66.40±1.36	31.53±7.58
	GFB-111 (50 mpk)	23.13±15.69	12.33±8.37
U87-MG	Control	23.48±6.21	15.68±3.25
"	GFB-111 (50 mpk)	10.50±2.12	6.00±1.13
"	GFB-111 (100 mpk)	7.73±2.61	5.20±1.71
"	GFB-111 (200 mpk)	5.13±3.10	3.07±1.94
C6	Control	20.23±7.84	9.77±3.13
	GFB-111 (50 mpk)	14.27±4.79	7.97±2.83
DaOY	Control	8.07±1.47	4.73±0.90
	GFB-111 (50 mpk)	7.20±2.52	4.80±1.37

9.9 Sprague Dawley Rat Study:

Vascular injury model

Sprague Dawley rats weighing 350 to 400 g are anesthetized using intraperitoneal pentobarbital (45mg/kg). The left common, internal and external carotid arteries may be exposed via a cervical collar incision. The left common carotid artery may be injured by the intraluminal passage of a 2F Fogarty catheter (Baxter Healthcare Corp. Santa Ana, CA) which is introduced via the external carotid artery inflated to 5 atm for 5 minutes. The external carotid artery may be ligated after removal of the catheter and the wound closed. Animals may be treated and cared for as recommended by the NIH Guide for the Care and Use of Laboratory Animals and the Animal Welfare Act.

Delivery of GFBs

At the time of carotid angioplasty, the contralateral jugular vein may be exposed and cannulated using PE 60 polyethylene tubing (Becton Dickinson, Sparks, MD). The catheter is tunneled subcutaneously to the back where it is attached to an Alzet miniosmotic pump (Alza Corporation, Palo Alto, CA). Pumps may be precalibrated to deliver either GFBs (12.5 to 100 mg/kg/day) or DMSO for 7 days after the balloon angioplasty procedure.

Morphometric analysis

Carotid arteries may be excised 14 days after injury and immediately fixed in 2% paraformaldehyde and dehydrated in 30% sucrose. Vessels may be then embedded in OCT medium. The arteries may be then cross-sectioned into 5µm sections taken from the center portion of the common carotid artery and stained using Mayer's hematoxylin and eosin stain. Intimal Thicknesses may be determined by measuring the distance from the luminal surface to the internal elastic lamina. Medial thicknesses may be determined by calculating the distance from the internal elastic lamina to the external elastic lamina. The intimal and medial layer thicknesses may be measured using the Optimas 3.0 imaging program (Optimas) and a Nikon scope (Nikon). These measurements may be used to determine the intima:media ratio (I/M). Statistical analysis may be performed using ANOVA with $p < 0.005$.

EXAMPLE 2 VEGF BINDING AGENTS

9.10 Inhibition of VEGF-Mediated Tyrosine Phosphorylation and MAP kinase Activation by GFB-116

In order to identify molecules that would bind vascular endothelial growth factor (VEGF), Flk-1/3T3 cell-based assay is used that relies on VEGF-stimulation of its receptor, Flk-1, tyrosine phosphorylation and subsequent MAP-kinase activation. The assay used herein is identical to that used for PDGF, except that NIH 3T3 cells that overexpress Flk-1 are used. GFB-116, which has four identical loops with the sequence GKGK is shown to be very potent and selective toward blocking the ability of VEGF to stimulate Flk-1 tyrosine phosphorylation and subsequent MAP-kinase activation as measured by phospho erk-1 and erk-2 also referred to in the Table as p42 and p44 (Table 4A). A preferred embodiment of the invention is a compound that binds to a vascular endothelial growth factor, of the general structure III (above) in which R is n-butyl, n-propyl, or benzyl, and X comprise covalently attached identical cyclic peptide loops wherein R₁, R₂, R₃, and R₄ are each amino acid side chains, as broadly defined above, that define a tetrapeptide sequence, wherein said tetrapeptide is covalently attached at the amino and carboxy termini of said tetrapeptide to 3-aminomethyl-5aminobenzamide linking group, and wherein said tetrapeptide sequence is GKGK, GDGY, or functionally related derivatives thereof.

Referring now to FIG.12, GFB-116 inhibits VEGF-stimulated flk-1 tyrosine phosphorylation with an IC_{50} value of 3mM . In contrast, GFB-116 does not inhibit PDGF-stimulated PDGFR tyrosine phosphorylation, even at concentrations as high as 10mM. This selectivity is further shown by administering GFB-116 to block VEGF-activation of the MAPKinases, Erk1 and Erk2, without affecting closely related growth factors (FIG.13). Furthermore, GFB-111 inhibits PDGF-stimulated MAPKinase activation whereas GFB-116 does not (FIG.13). The ability of GFB-116 to inhibit human tumor growth in nude mice is illustrated in FIG.14, which shows that GFB-116 at 50mg per Kg per day inhibits the growth of the human non-small cell lung carcinoma A-549 cell line in nude mice. Thus, GFB-116 is as potent as GFB-111 at inhibiting A-549 tumor growth (FIG.15). Using factor VIII immunostaining on tumors extracted from the above-described nude mice, demonstrates that GFB-116 is also efficacious at inhibiting angiogenesis *in vivo* (FIG.16). Quantification of the results by microvessel counting demonstrate that GFB-116 inhibits angiogenesis by 70% (Table 4B). Furthermore, GFB-116 was also effective at inhibiting the growth of B16-F10 melanoma in an immune-competent mouse model (Fig.16). A549 cells are implanted s.c. under the flank of nude mice. When the tumors reach an average size of about 150mm³, the animals are randomized and either treated with vehicle or S3I-124 (1mg/kg/day) for 10days. S3I-124 inhibits tumor growth by about 77%.

This embodiment of the present invention is a compound that binds to VEGF. Furthermore, the invention is directed to a composition for treating a subject, comprising the compound described immediately above, a pharmaceutically acceptable salt of this compound or combination thereof, in a pharmaceutically acceptable carrier.

A further embodiment of the invention is the administration of this composition to a patient having a condition comprising excess angiogenesis, a tumor or a combination thereof.

Table 4B. Inhibition of VEGF stimulated Flk-1 Tyrosine Phosphorylation and Signaling in Flk-1/3T3 by GFBs

Designation	Scaffold	R ₁ -R ₄ sequence	IC ₅₀ (μM)		
			Flk-1-Y-P	p42	p44
GFB-101	O4-Calix ^A	4-GFGD	>100	>100	>100
GFB-102	"	4-GDFD	100	>100	>100
GFB-103	"	4-GDGD			
GFB-104	O3-Calix ^A	4-GDGD			
GFB-105	But-Calix ^A	4-GDFD	<10	>100	>100

Designation	Scaffold	R ₁ -R ₄ sequence	IC ₅₀ (μM)		
			Flk-1-Y-P p42	p42	p44
GFB-106	"	4-GDDD	<10	<10	<10
GFB-107	"	4-GDGD	<10	100	100
GFB-108	"	4-d-ADGD ¹	10	50	50
GFB-109	"	4-GDLD			
GFB-110	"	4-GDAD			
GFB-111	"	4-GDGY	5	10	10
GFB-112	"	4-ADGD	<10	10	10
GFB-113	"	4-GDSD	<10	10	10
GFB-114	"	(4-) 8-acid (See Figure 7)			
GFB-115	"	4-GKGF		5 10	5 10
GFB-116	"	4-GK GK	0.3	5	5
GFB-117	"	4-GDND			
GFB-118	"	4-D-2-NalDGD ²			
GFB-119	"	4-PDGD			
GFB-120	"	4-GDDG			
GFB-121	"	4-d-AbuDGD ³			
GFB-122	"	4-GDDY			
GFB-123	"	1-GDDG			
GFB-124	"	See Figure 8			
GFB-126	"	3 x GDDG (See Figure 9)			
GFB-127	"	2 x GDDG (See Figure 9)			
GFB-128	"	DPM-2-GDGY (See Figure 10)			
GFB-129	"	DPM-2-GDDD (See Figure 10)			
GFB-130	"	DPM-2-GDDY (See Figure 10)			
GFB-131	"	DPM-2-GDDG (See Figure 10)			
GFB-132	But-Calix	3-GDGY-1-GDDG (See Figure 10)			
GFB-133	"	3-GDGY-1-CO ₂ H (See Figure 10)			
GFB-134	"	3-GDGY-1-GDDY (See Figure 10)			
GFB-135	"	3-GDGY-1-GDDD (See Figure 10)			
GFB-136	"	3-GDDD-1-GDGY (See Figure 10)			
GFB-137	"	2-GDGY-2-GDDG (See Figure 10)			

^A The difference between GFB-101 through GFB-103 (04-Calix), GFB-104 (03-Calix), and the rest of the GFB compounds (But-Calix) is related to the structure of the calix tail. (Please refer to Figure 6.)

¹ GFB-108: The "d" refers to D-amino acid, so d-ADGD is (D-Ala)-Asp-Gly-Asp.

² GFB-118: D-2 Nal is structurally related to D-Phe. Instead of the phenyl ring in Phe, it has a naphthelene ring linked through the 2-position.

³ GFB-121: d-Abu is D-aminobutyric acid. It has an ethyl group in the side chain while Ala has a methyl group in the side chain.

Table 4B: GFB-116 and GFB-111 Anti-angiogenic Activity *in vivo*

Tumor	Group	Microvessel Count (Mean \pm sd)	
		250X	400X
A-549	Control	13.58 \pm 4.44	9.52 \pm 3.08
	GFB-111 (50mpk)	5.68 \pm 1.74	3.58 \pm 1.01
	GFB-116 (50mpk)	5.56 \pm 1.75	3.46 \pm 1.19

EXAMPLE 3 aFGF BINDING AGENTS

9.11 Inhibition of aFGF Stimulation of p42/p44 by Growth Factor Binding Agents

The ability of growth factor binding agents in the library of growth factor binding agents to block an aFGF signal is measured by aFGF-stimulation of MAP kinase activation of p42 and p44 essentially as described above for VEGF. NIH 3T3 cells are used as the target cell. The growth factor binding molecules GFB-106, GFB-116, GFB-126, GFB-127, and GFB-133 are found to be very effective, see Table 5. A preferred embodiment of the invention is a compound that binds to acidic fibroblast growth factor, of the general structure III, in which R is n-butyl, n-propyl, or benzyl, and X₁, X₂, X₃, and X₄ optionally comprise covalently attached cyclic peptide loops of the general structure II, wherein R₁, R₂, R₃, and R₄ are each amino acid side chains that define a tetrapeptide sequence, wherein said tetrapeptide is covalently attached at the amino and carboxy termini of said tetrapeptide to a 3-aminomethyl-5-aminobenzamide linking group, and wherein said tetrapeptide sequence is as given, for example, in Table 5.

The present embodiment of the invention is directed to a compound that binds to a acidic fibroblast growth factor as described immediately above. Furthermore, the invention is directed to a composition for treating a subject comprising the compound described

immediately above, a pharmaceutically acceptable salt, or combination thereof, in a pharmaceutically acceptable carrier. A further embodiment of the invention is a method of use of the therapeutic composition described immediately above in which the composition is administered to a subject suffering from a condition comprising undesired angiogenesis, a tumor or a combination thereof.

Table 5. Inhibition of aFGF stimulated FGFR Tyrosine Phosphorylation and Signaling in NIH 3T3 by GFBs

Designation	Scaffold	R ₁ -R ₄ sequence	% Inhibition at 100 μ M		
			FGFR-Y-P	p42	p44
GFB-101	O4-Calix ^A	4-GFGD	ND		
GFB-102		4-GDFD	ND		
GFB-103		4-GDGD	ND		
GFB-104	O3-Calix ^A	4-GDGD	ND	2.5	0.7
GFB-105	But-Calix ^A	4-GDFD	ND	66.4	75.7
GFB-106		4-GDDD	ND	95.1	98.2
GFB-107		4-GDGD	ND	57.3	59.7
GFB-108		4-d-ADGD ¹	ND	67.8	80.1
GFB-109		4-GDLD	ND	68.3	79.7
GFB-110		4-GDAD	ND	76.1	85.2
				93.0	98.9
GFB-111		4-GDGY	ND	51.0	68.0
			ND	39.0	51.8
GFB-112		4-ADGD	ND	59.7	62.9
				40.2	39.6
GFB-113		4-GDSD	ND	53.6	64.3
GFB-114		(4-) 8-acid(See Figure 2)	ND	-8.7	-16.9
GFB-115		4-GKGF	ND	-21.0	-23.9
GFB-116		4-GK GK	ND	99.2	97.5
				99.6	97.2
				101.9	116.2
				99.7	99.9
				100.1 \pm 1.2	102.7 \pm 9.1
GFB-117		4-GDND	ND	63.4	80.8
				67.6	84.3
GFB-118		4-D-2-NalDGD ²	ND	20.6	30.5
GFB-119		4-PDGD	ND	62.6	78.9
			ND	72.1	81.7
				47.8	58.1
				60.8 \pm 12.2	72.9 \pm 12.9
GFB-120		4-GDDG	ND	68.1	74.3
GFB-121		4-d-AbuDGD ³	ND	8.7	8.9
GFB-122		4-GDDY	ND	57.3	71.6
GFB-123		1-GDDG	ND	11.4	12.5
GFB-124		(See Figure 3)	ND	0.3	-1.7
GFB-126		3 x GDDG (See Figure 4)	ND	96.5	106.0
GFB-127		2 x GDDG (See Figure 4)	ND	97.5	107.4
GFB-128		DPM-2-GDGY (See Figure 5)	ND	23.5	43.3

Designation	Scaffold	R ₁ -R ₄ sequence	% Inhibition at 100 μ M		
			FGFR-Y-P	p42	p44
GFB-129		DPM-2-GDDD (See Figure 5)	ND	11.3	3.2
GFB-130		DPM-2-GDDY (See Figure 5)	ND	78.9	78.9
GFB-131		DPM-2-GDDG (See Figure 5)	ND	33.4	44.1
GFB-132	But-Calix	3-GDGY-1-GDDG (See Figure 6)	ND	48.2	50.2
GFB-133		3-GDGY-1-CO ₂ H (See Figure 6)	ND	95.8	101.0
GFB-134		3-GDGY-1-GDDY (See Figure 6)	ND	54.8	64.1
GFB-135		3-GDGY-1-GDDD (See Figure 6)	ND	44.1	53.9
GFB-136		3-GDDD-1-GDGY (See Figure 6)	ND	73.1	84.5
GFB-137		2-GDGY-2-GDDG (See Figure 6)	ND	6.4	19.9

^A The difference between GFB-101 through GFB-103 (04-Calix), GFB-104 (O3-Calix), and the rest of the GFB compounds (But-Calix) is related to the structure of the calix tail. (Please refer to Figure 1.)

¹ GFB-108: The "d" refers to D-amino acid, so d-ADGD is (D-Ala)-Asp-Gly-Asp.

² GFB-118: D-2 Nal is structurally related to D-Phe. Instead of the phenyl ring in Phe, it has a naphthalene ring linked through the 2-position.

³ GFB-121: d-Abu is D-aminobutyric acid. It has an ethyl group in the side chain while Ala has a methyl group in the side chain.

EXAMPLE 4 IGF-1 BINDING AGENTS

9.12 Inhibition of Insulin-like Growth Factor-1 (IGF-1) Mediated Tyrosine Phosphorylation and MAP Kinase Activation by GFB-126

The ability of the GFB library to block the ability of IGF-1 to stimulate the IFG-1 R tyrosine phosphorylation and MAP kinase activation is evaluated using the cell assay described above with IGF-1R/3T3 cells as target cell. IFG-1R/3T3 cells are cells transfected with the IGF-1R gene to overproduce IFG-1R. IFG-1R has been shown to be critical to survival of many human cancer cell types and for tumor progression. GFB-126 is found to be particularly effective in blocking IFG-1 action, see Table 6. A preferred embodiment of the invention is a compound that binds to an insulin-like growth factor-1, of the general structure III, wherein R is n-butyl, n-propyl, or benzyl, and X₁, X₂, X₃, and X₄ optionally comprise identical cyclic

peptide loops of the general structure II, and wherein R₁, R₂, R₃, and R₄ are each amino acid side chains that define a tetrapeptide sequence, wherein said tetrapeptide is covalently attached at the amino and carboxy termini of said tetrapeptide to a 3-aminomethyl-5-aminobenzamide linking group.

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This invention is also directed to a compound that binds to an insulin-like growth factor-1 as described immediately above. The invention is further directed to a composition for treating a subject, comprising administering the compound described immediately above, a pharmaceutically acceptable salt of this compound, or combination thereof, in a pharmaceutically acceptable carrier, and in which the subject suffers excess cellular proliferation, excess angiogenesis, a tumor, or a combination thereof.

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Table 6. Inhibition of IGF-1 stimulated IGFR Tyrosine Phosphorylation and Signaling in IGFR/3T3 by GFB's

Designation	Scaffold	R ₁ -R ₄ sequence	% Inhibition at 100 μ M IGF1R-Y-P		
			p42	p44	
GFB-101	O4-Calix ^A	4-GFGD	ND		
GFB-102	"	4-GDFD	ND		
GFB-103	"	4-GDGD	ND		
GFB-104	O3-Calix ^A	4-GDGD	-28.4	-0.19	22.4
GFB-105	But-Calix ^A	4-GDFD	1.3	16.1	37.0
GFB-106	"	4-GDDD	ND	ND	ND
GFB-107	"	4-GDGD	ND	ND	ND
GFB-108	"	4-d-ADGD ¹	ND	ND	ND
GFB-109	"	4-GDLD	ND	ND	ND
GFB-110	"	4-GDAD	40.9 32.4	27.2 8.6	55.4 47.1
GFB-111	"	4-GDGY	-12.4	-18.4	10.7
GFB-112	"	4-ADGD	0.6	-7.6	30.4
GFB-113	"	4-GDSD	-18.9	-31.4	18.7
GFB-114	"	(4-) 8-acid (See Figure 7)	-28.9	43.6	76.8
GFB-115	"	4-GKGF	-38.3	-12.4	51.9
GFB-116	"	4-GK GK	-149.2 -90.0	-29.8 5.2	25.2 43.5
GFB-117	"	4-GDND	4.9	17.1	61.5
GFB-118	"	4-D-2-NalDGD ²	1.5	16.4	55.2
GFB-119	"	4-PDGD	16.7	29.7	66.2

Designation	Scaffold	R ₁ -R ₄ sequence	% Inhibition at 100μM		
			IGF1R-Y-P	p42	p44
GFB-120	"	4-GDDG	35.4	68.1	85.9
GFB-121	"	4-d-AbuDGD ³	33.8	28.3	62.1
GFB-122	"	4-GDDY	38.4	33.6	66.6
GFB-123	"	1-GDDG	24.2	26.7	62.4
GFB-124	"	See Figure 8	-9.5	3.0	-20.4
GFB-126	"	3 x GDDG (See Figure 9)	51.7	63.9	71.2
			51.0	47.0	79.2
GFB-127	"	2 x GDDG (See Figure 9)	36.8	38.4	61.6
			31.8	27.2	74.4
GFB-128	"	DPM-2-GDGY (See Figure 10)	-12.0	-6.1	38.2
GFB-129	"	DPM-2-GDDD (See Figure 10)	3.4	2.8	33.4
GFB-130	"	DPM-2-GDDY (See Figure 10)	22.7	-19.7	-17.5
GFB-131	"	DPM-2-GDDG (See Figure 10)	30.2	-6.2	20.0
GFB-132	But-Calix	3-GDGY-1-GDDG (See Figure 10)	30.2	11.0	39.4
GFB-133	"	3-GDGY-1-CO ₂ H (See Figure 10)	ND	ND	ND
GFB-134	"	3-GDGY-1-GDDY (See Figure 10)	16.8	4.9	53.5
GFB-135	"	3-GDGY-1-GDDD (See Figure 10)	16.2	0.8	46.1
GFB-136	"	3-GDDD-1-GDGY (See Figure 10)	40.6	15.0	59.5
GFB-137	"	2-GDGY-2-GDDG (See Figure 10)	36.0	5.9	38.2

^A The difference between GFB-101 through GFB-103 (O4-Calix), GFB-104 (O3-Calix), and the rest of the GFB compounds (But-Calix) is related to the structure of the calix tail. (Please refer to Figure 6.)

¹ GFB-108: The "d" refers to D-amino acid, so d-ADGD is (D-Ala)-Asp-Gly-Asp.

² GFB-118: D-2 Nal is structurally related to D-Phe. Instead of the phenyl ring in Phe, it has a naphthalene ring linked through the 2-position.

³ GFB-121: d-Abu is D-aminobutyric acid. It has an ethyl group in the side chain while Ala has a methyl group in the side chain.

The above examples have been depicted solely for the purpose of exemplification and are not intended to restrict the scope or embodiments of the invention. Other embodiments not specifically described should be apparent to those of ordinary skill in the art. Such other

embodiments are considered to fall, nevertheless, within the scope and spirit of the present invention. Thus, the invention is properly limited solely by the claims that follow.

100 101 102 103 104 105 106 107 108 109 110 111 112 113 114 115 116 117 118 119 120 121 122 123 124 125 126 127 128 129 130 131 132 133 134 135 136 137 138 139 140 141 142 143 144 145 146 147 148 149 150 151 152 153 154 155 156 157 158 159 160 161 162 163 164 165 166 167 168 169 170 171 172 173 174 175 176 177 178 179 180 181 182 183 184 185 186 187 188 189 190 191 192 193 194 195 196 197 198 199 200 201 202 203 204 205 206 207 208 209 210 211 212 213 214 215 216 217 218 219 220 221 222 223 224 225 226 227 228 229 230 231 232 233 234 235 236 237 238 239 240 241 242 243 244 245 246 247 248 249 250 251 252 253 254 255 256 257 258 259 260 261 262 263 264 265 266 267 268 269 270 271 272 273 274 275 276 277 278 279 280 281 282 283 284 285 286 287 288 289 290 291 292 293 294 295 296 297 298 299 300 301 302 303 304 305 306 307 308 309 310 311 312 313 314 315 316 317 318 319 320 321 322 323 324 325 326 327 328 329 330 331 332 333 334 335 336 337 338 339 340 341 342 343 344 345 346 347 348 349 350 351 352 353 354 355 356 357 358 359 360 361 362 363 364 365 366 367 368 369 370 371 372 373 374 375 376 377 378 379 380 381 382 383 384 385 386 387 388 389 390 391 392 393 394 395 396 397 398 399 400 401 402 403 404 405 406 407 408 409 410 411 412 413 414 415 416 417 418 419 420 421 422 423 424 425 426 427 428 429 430 431 432 433 434 435 436 437 438 439 440 441 442 443 444 445 446 447 448 449 450 451 452 453 454 455 456 457 458 459 460 461 462 463 464 465 466 467 468 469 470 471 472 473 474 475 476 477 478 479 480 481 482 483 484 485 486 487 488 489 490 491 492 493 494 495 496 497 498 499 500 501 502 503 504 505 506 507 508 509 510 511 512 513 514 515 516 517 518 519 520 521 522 523 524 525 526 527 528 529 530 531 532 533 534 535 536 537 538 539 540 541 542 543 544 545 546 547 548 549 550 551 552 553 554 555 556 557 558 559 560 561 562 563 564 565 566 567 568 569 570 571 572 573 574 575 576 577 578 579 580 581 582 583 584 585 586 587 588 589 590 591 592 593 594 595 596 597 598 599 600 601 602 603 604 605 606 607 608 609 610 611 612 613 614 615 616 617 618 619 620 621 622 623 624 625 626 627 628 629 630 631 632 633 634 635 636 637 638 639 640 641 642 643 644 645 646 647 648 649 650 651 652 653 654 655 656 657 658 659 660 661 662 663 664 665 666 667 668 669 670 671 672 673 674 675 676 677 678 679 680 681 682 683 684 685 686 687 688 689 690 691 692 693 694 695 696 697 698 699 700 701 702 703 704 705 706 707 708 709 710 711 712 713 714 715 716 717 718 719 720 721 722 723 724 725 726 727 728 729 730 731 732 733 734 735 736 737 738 739 740 741 742 743 744 745 746 747 748 749 750 751 752 753 754 755 756 757 758 759 760 761 762 763 764 765 766 767 768 769 770 771 772 773 774 775 776 777 778 779 780 781 782 783 784 785 786 787 788 789 790 791 792 793 794 795 796 797 798 799 800 801 802 803 804 805 806 807 808 809 810 811 812 813 814 815 816 817 818 819 820 821 822 823 824 825 826 827 828 829 830 831 832 833 834 835 836 837 838 839 840 841 842 843 844 845 846 847 848 849 850 851 852 853 854 855 856 857 858 859 860 861 862 863 864 865 866 867 868 869 870 871 872 873 874 875 876 877 878 879 880 881 882 883 884 885 886 887 888 889 890 891 892 893 894 895 896 897 898 899 900 901 902 903 904 905 906 907 908 909 910 911 912 913 914 915 916 917 918 919 920 921 922 923 924 925 926 927 928 929 930 931 932 933 934 935 936 937 938 939 940 941 942 943 944 945 946 947 948 949 950 951 952 953 954 955 956 957 958 959 960 961 962 963 964 965 966 967 968 969 970 971 972 973 974 975 976 977 978 979 980 981 982 983 984 985 986 987 988 989 990 991 992 993 994 995 996 997 998 999 1000